

The evolution of worker sterility in honeybees: the genetic basis of failure of worker policing

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Worker honeybees (*Apis mellifera*) usually only lay eggs when their colony is queenless. However, an extremely rare 'anarchistic' phenotype occurs, in which workers develop functional ovaries and lay large numbers of haploid eggs which develop into adult drones despite the presence of the queen. Studies of such colonies can give important insights into the mechanisms by which worker sterility is maintained in normal colonies. Here we report on the results of a breeding programme which enhanced the frequency of the anarchistic phenotype. Colonies derived from queens inseminated only by worker-laid males showed up to 9% of workers with highly developed ovaries. In these colonies a large proportion of males arose from worker-laid eggs. Colonies headed by queens inseminated with 50% worker-laid drones and 50% queen-laid drones showed variable phenotypes. In most such colonies there was no worker reproduction. In some, many workers had highly developed ovaries, but no worker-laid eggs were reared. In one colony, many worker-laid eggs were reared to maturity. The results suggest that the anarchy phenotype results from a complex interaction of queen genotype, the worker genotype of subfamilies that successfully reproduce and of those that do not, and the external environment.

Keywords: worker policing; anarchy; social insects; kin selection; *Apis mellifera*; laying workers

1. INTRODUCTION

Kin selection theory (Hamilton 1964) predicts that, in polyandrous, monogynous social insect colonies, reproductive conflicts should arise both among workers and between workers and the queen over the maternity of the colony's males (Ratnieks 1988; Ratnieks & Reeve 1992). A worker is most related to her own son ($r=0.5$), then to the son of a super-sister ($r=0.375$), then to the son of her maternal queen ($r=0.25$) and least to the son of a half-sister ($r=0.125$) (Page & Erickson 1988; Ratnieks 1988). Workers should therefore 'prefer' to rear their own male eggs rather than those of the queen. However, such worker reproduction is the worst option for other workers because they are so distantly related to the sons of half-sisters (Ratnieks 1988; Ratnieks & Reeve 1992). An evolutionary compromise exists in which workers forgo personal reproduction and rear sons of the queen. However, theory also predicts the evolution of 'cheating' behaviour, in which workers contribute (male) eggs into the general pool laid by the queen. A further prediction (Ratnieks 1988; Ratnieks & Reeve 1992) is that workers should try to prevent this cheating since, on average, queen eggs are more related to them than eggs laid by another worker. This is known as the 'worker policing' hypothesis (Ratnieks 1988; Ratnieks & Visscher 1989; Ratnieks & Reeve 1992; Visscher 1996).

There is strong evidence that worker policing has evolved in *Apis mellifera*. In normal colonies around 10% of workers show some signs of ovary development such as minor swelling of the ovarioles (Jay 1968, 1970;

Kropacova & Haslbachova 1969; Visscher 1996) and around 0.01% of workers actually lay eggs. However, although *ca.* 7% of male eggs are laid by these few workers, 99.88% of these eggs are removed by worker policing (Visscher 1996).

The existence of successful cheating behaviour by workers has also been demonstrated. In certain rare colonies, the majority of drones are the offspring of workers rather than the queen (Page & Erickson 1988; Oldroyd *et al.* 1994; Montague & Oldroyd 1998). This behaviour has been termed 'anarchistic' because it represents a failure of effective policing (Oldroyd *et al.* 1994).

Anarchistic behaviour is not an epiphenomenon of queenlessness (Montague & Oldroyd 1998). When honeybees become queenless, some workers develop active ovaries and develop as false queens (Sakagami 1958; Robinson *et al.* 1990; Van der Blom 1991; Page & Robinson 1994). There is considerable genetic variation in the speed at which workers develop ovaries and commence egg laying and the degree to which they tolerate worker-laid eggs (Robinson *et al.* 1990; Page & Robinson 1994; Visscher & Dukas 1995; Moritz *et al.* 1996). However, Montague & Oldroyd (1998) showed that, when an anarchistic colony was made queenless, the subfamily that was actively egg laying when the colony was queenright did not contribute significantly to worker reproduction when the colony was queenless. Moreover, they showed that anarchistic workers can be active in combs regularly patrolled and used by the queen for egg laying. Thus, anarchistic behaviour appears to be a fundamentally different behaviour to worker oviposition in queenless colonies. It is absolutely not an artefact of the use of queen excluders, as has been suggested (Visscher 1998).

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Montague & Oldroyd (1998) suggested that the anarchy phenotype requires two separate behavioural mutations. First, anarchistic workers must overcome substances secreted by the queen and brood that normally inhibit the development of worker ovaries (Free 1987). Second, workers of this subfamily must escape the worker policing which normally ensures worker reproduction is minimized (Ratnieks 1988, 1995; Ratnieks & Visscher 1989; Ratnieks & Reeve 1992; Visscher 1996).

We investigated the degree to which genes regulate the anarchistic phenotype with a selective breeding programme using worker-laid ('anarchistic') males as fathers for two generations. We also examined the phenotype of colonies derived from queens inseminated with both anarchistic males and queen-laid, wild-type males and compared this to the phenotype of colonies derived from queens inseminated solely by anarchistic males. These investigations help in the understanding of the behavioural modifications required to generate the 'anarchistic' phenotype. More generally, they provide insights into the evolution of worker sterility and worker policing.

2. METHODS

The anarchistic colony reported by Montague & Oldroyd (1998) was used to initiate the selection programme. The queen had been naturally inseminated by at least 20 males. Daughters of just one of these males were the mothers of *ca.* 80% of the male offspring produced by this colony.

The overall selection strategy is illustrated in figure 1. In October 1995 we reared daughter queens from the original colony and mated them to their worker-laid nephews. Colonies developed in the spring of 1996 to varying degrees, so that most were very strong and produced many males. In November 1996, daughter queens were reared from the most anarchistic F_1 colony and mated with worker-laid males from another anarchistic F_1 colony.

For both generations, we did not inseminate all queens with worker-laid males alone. Rather, for each generation some daughter queens were inseminated with one or several (probably brother) wild-type drones caught at the entrance of colonies not showing anarchistic behaviour and one or several anarchistic (worker-laid) drones. The drones used for insemination were frozen at -70°C .

From September 1997 to March 1998, the second generation colonies were examined at regular intervals for the presence of anarchistic behaviour (eggs, larvae or pupae found in drone combs above the queen excluder) and worker bees and drone brood were sampled as appropriate. All colonies developed to the extent that they were producing males in large numbers.

To determine the paternity or maternity of an individual, we used microsatellite analysis using the honeybee microsatellite loci A88 or A107 identified by Estoup *et al.* (1994).

3. RESULTS

(a) Selection programme

Of the queens inseminated in November 1995, five colonies headed by queens inseminated only by anarchistic drones and six colonies headed by queens inseminated with both anarchistic and wild-type drones survived to October 1996. Only those colonies in which the queen had been exclusively inseminated

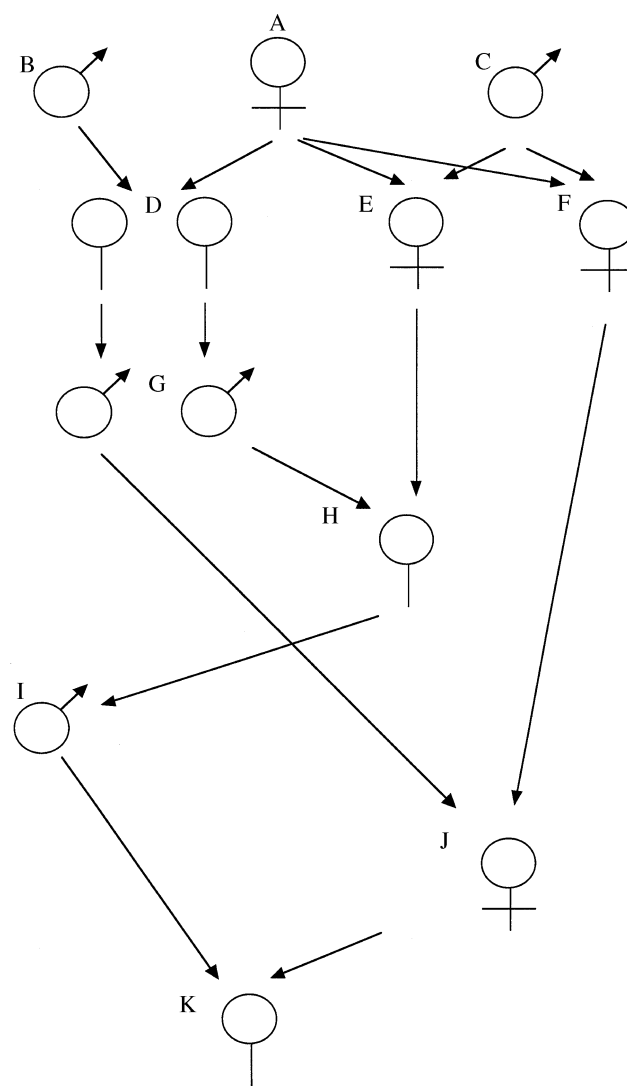


Figure 1. Overall breeding strategy to obtain anarchistic colonies. We follow the standard nomenclature and symbols for haplodiploid pedigrees (e.g. Polhemus *et al.* 1950). Queen A was identified by widespread advertisement and shown (Montague & Oldroyd 1998) to have been inseminated by one anarchistic male (drone B) and several wild-type males (e.g. drone C). This colony was simply line bred for two generations. To do this, a daughter, queen E, was reared from queen A and inseminated with her worker-laid nephews such as drone G. Worker daughters of this mating such as H laid unfertilized eggs to produce sons such as drone I at high frequency. Daughter queens were reared from queen F and inseminated with drone I males. Daughter workers (K) of the $I \times J$ mating produced offspring males at high frequency.

with anarchistic males exhibited anarchistic behaviour (table 1).

Of the second generation queens inseminated in November 1996, both of the two colonies inseminated only with worker-laid males exhibited the anarchistic phenotype (table 2). Between 5% and 9% of workers sampled off drone comb in these colonies had highly developed ovaries containing mature eggs (table 3). Out of the 11 queens inseminated with 50% worker-laid drones and 50% wild-type drones, one exhibited the anarchistic phenotype very strongly, a second weakly and a third very weakly (table 2).

Table 1. *Presence or absence of anarchy phenotype in colonies of honeybees in which queens were inseminated with varying proportions of worker-laid males: generation 1 (inspection September 1996)*

queen	number of worker-laid inseminating males	number of queen-laid inseminating males	phenotype	comments
B55	10	0	> 500 worker-laid pupae	—
B73	1	0	> 500 worker-laid pupae	—
B91	10	0	six worker-laid pupae	—
B76	10	0	no worker oviposition	—
B97	10	0	no worker oviposition	colony too weak to rear drones; colony was weakly anarchistic in following season
B33	1	1	no worker oviposition	colony too weak to rear drones
B24	1	1	five worker-laid eggs	colony queenless at time of inspection
three colonies	1	1	no worker oviposition	—
four colonies	4	4	no worker oviposition	—

Table 2. *Presence or absence of anarchy phenotype in colonies of honeybees in which queens were inseminated with varying proportions of worker-laid males for generation 2 (inspection 4 December 1997)*

queen	number of worker-laid inseminating males	number of queen-laid inseminating males	phenotype
W75	5	5	> 500 worker-laid brood
seven colonies	5	5	no worker oviposition
W34	5	5	14 worker-laid pupae
W89	5	5	one worker-laid pupa
W77	10	0	> 500 worker-laid brood
W84	10	0	> 500 worker-laid brood

Workers were taken from the drone comb of two second-generation colonies (W74 and W53) which had not exhibited anarchistic behaviour (i.e. no eggs were observed in drone combs inaccessible to the queen) at any time (table 2). These colonies had been inseminated with 50% wild-type and 50% anarchistic males. No workers showed ovary development sufficient to permit oviposition in colony W74, whereas 7.4% of workers were judged to be laying workers in colony W53 (table 3). Thus, in colony W53 it is very likely that many workers were laying, but that their eggs were being efficiently policed.

Microsatellite analysis of 188 dissected workers from queen W53 showed that 29% were fathered by anarchistic males and 71% were fathered by wild-type males. Of the 14 workers with developed ovaries, 42% were fathered by anarchists and 51% by wild-type males. Therefore, in this colony the probability that a worker would develop functioning ovaries was not affected by paternity ($\chi^2=1.35$, d.f. = 1 and $p>0.2$) as the ratio of wild-type to anarchist fathers did not differ from the ratio of worker offspring with developed ovaries.

Table 3. *Analysis of worker ovary development in six colonies headed by queens inseminated by anarchistic males*

queen	date workers collected	workers with eggs in ovaries	workers without eggs in ovaries
W84	11 November 1997	6	94
W84	30 March 1998	5	95
W77	14 November 1997	9	91
W74	14 November 1997	0	100
W34	2 February 1997	0	100
W75	5 February 1998	8	246
W53	14 November 1997	14	188

Workers were taken from the surface of drone combs of the two genetically mixed colonies (W34 and W75) that displayed anarchistic behaviour. No workers with highly developed ovaries were detected in colony W34 which had only 14 drone pupae laid by workers, whereas 3.2% of workers taken from the drone comb had highly developed ovaries in the highly anarchistic colony W75 (table 3).

Genetic analysis of colony W75 showed that, out of the 246 workers dissected, 48% were sired by anarchist males and 52% by wild-type males. Of the eight physiogastric workers, seven were fathered by anarchist males, while one was fathered by a wild-type male. In this colony therefore, paternity was strongly correlated with the development of ovaries in workers ($\chi^2=5.2$, d.f. = 1 and $p=0.02$) and the wild-type subfamilies were unable to police the eggs laid effectively.

Out of the 73 drone pupae analysed from colony W75, 14 were produced by daughters of wild-type males, while the remainder (59) were grandsons of the anarchistic males used in the insemination.

Thirteen worker-laid males were recovered in colony W34 and microsatellite analysis suggested that all these were grandsons of anarchist males, although the distribution of alleles meant that a small number could have been grandsons of wild-type males. Thus, in colonies W34 and W75, paternity affected both rates of ovary development and probably egg survival as well.

Table 4. *Predictions about worker phenotypes under various models of genetic control of the anarchy phenotype*

scenario	predicted outcome		
	worker ovary development	paternity of adult drones	plausible genetic cause
(i) general failure of worker sterility	all subfamilies	all subfamilies	mutation in queen reducing signals to workers
(ii) general failure of worker sterility, but policing fails only in some subfamilies	all subfamilies	selected subfamilies	mutation in queen reduces sterility signals to workers, but eggs from non-anarchistic subfamilies are policed
(iii) general failure of worker policing, but ovary development only in selected subfamilies	selected subfamilies	selected subfamilies	mutation in queen reduces policing; mutation in one male allows sterility signals from queen and brood to be overridden
(iv) failure of worker policing and ovary suppression in one subfamily	one subfamily	one subfamily	mutation(s) in one male allow daughters to develop ovaries and eggs to escape policing

4. DISCUSSION

This study has demonstrated that the anarchistic phenotype is highly heritable, since the majority of colonies headed by queens inseminated solely with worker-laid drones exhibited the anarchistic phenotype. The variable phenotypes of the colonies headed by queens inseminated with both anarchistic and wild-type drones suggests that control of worker sterility is extremely complex. Variables which apparently interact to determine whether workers successfully reproduce include (i) queen genotype, (ii) the genotype of all subfamilies (not only anarchistic ones), and (iii) the external environment.

Montague & Oldroyd (1998) postulated that the anarchy phenotype could arise in one of four ways (table 4). The data from the current study does not provide any support for scenario (i) (table 4) since survival of worker-laid eggs was shown to be dependent on worker paternity in a number of the anarchistic colonies. However, the variable expression of the anarchy phenotype across these colonies does not provide exclusive support for any of the remaining three scenarios. It appears that anarchistic behaviour can arise via a variety of routes. In support of this notion, we discuss each group of colonies in turn.

(a) *First generation colonies*

In the first generation of selection, two of the five queens inseminated exclusively with semen obtained from anarchistic males were strongly anarchistic, one was weakly anarchistic and two showed no signs of worker oviposition. Out of the six queens inseminated with a mixture of anarchistic and wild-type males, no colonies developed the anarchistic phenotype (table 1). These results suggest that the presence of a wild-type subfamily within the colony prevented the development of the anarchy phenotype. A likely cause of this is that some daughters of the anarchist males were laying, but that these eggs were recognized by members of the wild-type subfamilies as being worker laid and eaten. In the colonies which did not contain wild-type subfamilies, policing was less effective. This strongly suggests that wild-type subfamilies are more effective at policing than anarchistic

subfamilies. However, we cannot exclude other possibilities. First, the presence of wild-type workers may have prevented the development of ovaries in the daughters of the anarchist males. Second, as the anarchistic subfamilies were highly related, one might postulate that anarchistic subfamilies showed reduced levels of policing because they were more closely related to the eggs being laid than were the wild-type subfamilies.

(b) *Second generation colonies*

In the second generation, two out of the eight colonies headed by queens inseminated with both wild-type and anarchistic males displayed the anarchy phenotype, and one of these strongly so. Both of the two colonies inseminated exclusively with worker-laid males were strongly anarchistic with 6–9% of workers showing highly developed ovaries and large numbers of worker-laid males present in the colonies. Thus, these second generation results again suggest that the presence of wild-type subfamilies usually suppresses worker reproduction. However, the expression was variable. Workers from colony W74 showed no signs of ovary development and no signs of reproduction. Some daughters of both anarchistic and non-anarchistic drones showed high levels of ovary development in colony W53, but either these workers were not laying or (more likely) the eggs laid were effectively policed. Workers with developed ovaries were much more likely to be daughters of anarchistic males in colony W75 and the males produced by this colony were overwhelmingly grandsons of anarchistic males. Although no workers with developed ovaries were detected in colony W34, all worker-laid drones were grandsons of anarchistic males. Therefore, although the numbers of workers with active ovaries were too small for useful comparisons with the frequency of offspring males, we suggest that eggs laid by daughters of anarchistic males were more likely to be reared than those laid by the daughters of wild-type males.

(c) *General considerations*

Selection for the anarchistic phenotype appears to have relaxed suppression of ovary development in workers in

all subfamilies. This may have occurred because the queen and brood of our second generation colonies produced less ovary-inhibiting substances than are produced in normal colonies. However, this conclusion should be treated with some caution for three reasons. First, although we have demonstrated an extraordinarily high rate of ovary development (up to 9% versus around 0.01% in previous studies), these earlier studies (Ratnieks 1993; Visscher 1996) were not conducted under the same conditions as ours. Visscher's study (1996) was conducted in July to August, when day length contracts and environmental conditions are less conducive to maximum brood rearing. Ratnieks' (1993) study was conducted all year round, so that some samples were obtained in winter and late summer. Perhaps Ratnieks (1993) and Visscher (1996) would have found higher rates of ovary development if they had made collections exclusively in spring when brood nests were expanding rapidly. Second, our collections were made from the drone comb in an attempt to maximize the proportion of workers with developed ovaries. Thus, the rates of worker oviposition we detected might have been higher than if we had taken a more random samples of bees.

We conclude that our selection programme (i) strongly increased rates of worker ovary development, (ii) probably led to an increase in tolerance of the effects of inhibitory pheromones produced by the queen in the workers sired by anarchists, and (iii) maintained or increased survival rates of worker-laid eggs laid by workers of anarchistic subfamilies. Thus, it appears that the anarchy phenotype can arise via several routes which include elements from all the scenarios outlined above. Selection as performed by us may have caused the expression of a number of factors to alter simultaneously. Daughters of wild-type males probably acquired anarchistic tendencies via their mother, who was derived from anarchistic stock. This may explain why daughters of wild-type males had well-developed ovaries in colony W53 and tolerated eggs laid by daughters of non-anarchistic males in colony W75. A critical experiment therefore, is to inseminate wild-type queens with a single anarchistic male and a number of unrelated wild-type males and to then determine the relative survival of their eggs (i) if only daughters of the anarchistic male develop ovaries, and (ii) if both wild-type and anarchistic daughters develop ovaries.

Finally, we reiterate that worker reproduction in queen-right colonies is a rare but easily selectable phenomenon. One criticism of the worker policing hypothesis (Woyciechowski & Lomnicki 1987; Ratnieks 1988; Ratnieks & Visscher 1989) is that, in order for worker policing to be selected, worker oviposition must occur, if not ubiquitously then at least occasionally. Our study has shown that worker reproduction occurs in honeybees and can be a very significant component of a colony's reproductive output. Thus, the necessary selective force for the evolution of worker policing is apparent.

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